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**Cloning and characterization of the calcineurin A gene
of *Schizosaccharomyces pombe***

by



Dorota Plochocka-Zulinska

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements
for the degree of
Master of Science

DEPARTMENT OF ANATOMY AND CELL BIOLOGY

Edmonton, Alberta
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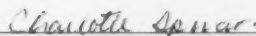
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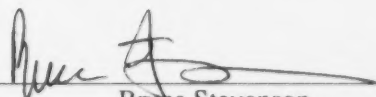
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Colin Rasmussen



Charlotte Spencer



Bruce Stevenson

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I dedicate this thesis to my Lord and Saviour Jesus Christ and to all the rest of my family who have supported me, believed in me and stood by me in spite of my failures.

I am also greatly indebted to Dr. Colin Rasmussen, Grace Rasmussen and Graig Garen.

Without their dedication I would not have been able to accomplish this work.

Abstract

Primers constructed on the basis of conserved regions present in all calcineurin A (CnA) gene products were used in PCR reactions with *S. pombe* genomic DNA as a template to obtain a CnA probe. The entire *S. pombe* CnA gene was screened from each of: λ bacteriophage Fix II and an ordered P_1 bacteriophage *S. pombe* genomic library with the CnA PCR probe. Based on the P_1 library screening results, the chromosomal locus of CnA gene was located between *cdc10* and *top1* genes on chromosome II. The cloned CnA gene is 1865 bp long and has two introns, 45 and 155 bp long respectively. The putative gene product consists of 554 amino acids. The predicted protein sequence encoded by the CnA gene has 57% identity to rat CnA2, 51% to human CnA2 and 45% to the *S. cerevisiae* CnA1 gene products respectively. A putative TR cis-acting regulatory element (TTCTTTGTTY) for the *ste11* transcription factor, matching at 9/10 positions, was identified in the promoter of CnA gene, -137 nucleotides relative to the initiation methionine codon. Block/release cell cycle experiments, performed with a temperature-sensitive *cdc 25-22* strain of *S. pombe* have shown that CnA mRNA levels vary in cell cycle dependent manner, reaching peak levels during S phase. CnA mRNA levels examined in an *S. pombe* wild type strain under various nutritional conditions have shown CnA mRNA levels are most highly expressed in cells starved for nitrogen (8-fold higher than in exponential cells) and in cells grown to stationary phase (4-fold higher than in exponential cells). Maximal levels of CnA mRNA in *S. pombe* wild type cells occurred within 8 hr following the shift to nitrogen free medium and remained at this peak for at least 25 hr. Addition of extracellular caffeine and cAMP in nitrogen limiting medium inhibited this induction. CnA mRNA levels also remained uninduced during nitrogen starvation of *S. pombe* strain lacking the *ste11* gene. These results indicate that in *S. pombe*, CnA gene expression is regulated by the *ste11* transcription factor. This pathway, typical for activation of genes involved in mating response, points to the role of CnA in *S. pombe* sexual differentiation.

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Introduction

In order to reproduce every cell must undergo a series of events known as the cell cycle. Cells may also withdraw from the cell cycle and enter a non-proliferative state, from which they may differentiate or resume proliferation depending on environmental conditions. In somatic cells the cell cycle can be divided into M-phase and interphase. The M-phase consists of nuclear and cytoplasmic division, known as mitosis and cytokinesis respectively. Interphase is composed of G_1 , S and G_2 phases and normally accounts for most of the total cell cycle time. The G_1 phase separates M-phase and S-phase. The S phase is the period when DNA is replicated. The G_2 phase separates S from M-phase and is the time when cells prepare for mitosis and cytokinesis (reviewed by Heichman and Roberts, 1994).

During G_1 phase a decision is made, in which a cell commits to undergo DNA synthesis and to proceed to mitosis, or exit the cell cycle and enter a quiescent state called G_0 . Commitment to proliferate has been called Start in unicellular organisms such as yeast, or the restriction point (R) in mammalian cells (Johnston et al., 1977; Nurse and Bisset, 1981; Pardee, 1974). A second checkpoint takes place during G_2 phase. Cells with undamaged and duplicated DNA pass the G_2 /M transition and undergo mitosis, but cells with DNA damage arrest in G_2 while repair mechanisms operate (Murray, 1992). A third cell cycle checkpoint exists in mitosis, at the metaphase/anaphase transition. The existence of these checkpoints assures proper coordination of the cell growth and nuclear division cycles. In the last twenty years, biochemical investigations in both vertebrate and invertebrate species, coupled with genetic studies in yeast have identified many regulatory proteins that control progression at each of these decision points. What has become clear is that the control of cell cycle events is a result of complex coordinate regulation of multiple biochemical pathways.

Growth and proliferation of the cells are strongly influenced by many extracellular signals. It is known that mating factors or nutrient access affect the ability of unicellular organisms

cells, like fungi and yeast, to complete the Start function (reviewed by Obara-Ishihara and Okayama, 1994). It shows that animal cells require stimulation of polypeptide growth factors in order to proliferate (Brooks, 1976; Larsson et al., 1986). These extracellular stimuli are converted through many signal transduction pathways into intracellular signals, which further affect cell growth and proliferation.

The role of intracellular signaling molecules like cAMP and calcium in regulating cellular events has been well characterized. Second messengers, like cAMP and calcium act via protein intermediates which further regulate the activity of a diverse collection of intracellular proteins (Mellow et al., 1989; Gonzales and Montminy, 1989).

Calcium is involved in many cell cycle events. This cation has been shown to be essential in proliferation of sea urchin embryos and cultured animal cells, being required for G₁/S progression (Hazelton et al., 1979; Whitfield et al., 1976), G₂/M transition and during mitosis, for nuclear envelope breakdown, chromatin condensation, and chromosome movement at the metaphase/anaphase transition (Keith et al., 1985; Poenie et al., 1985). In addition Ca²⁺ is required for quiescent cells to reenter the cell cycle (reviewed by Means, 1994). Calcium acts through Ca²⁺-binding proteins such as protein kinase (PK-C) and the ubiquitous intracellular Ca²⁺ receptor calmodulin (CaM).

The crystal structure of calmodulin reveals that this protein consists of two globular domains separated by a central α -helical linker domain. Each globular domain contains two Ca²⁺ binding motifs known as "EF hands" (reviewed by Means et al., 1991). Together with troponin C and parvalbumin, CaM belongs to the superfamily of EF-hand Ca²⁺-binding proteins (Kretsinger, 1980). Calmodulin is a primary mediator of Ca²⁺-dependent signaling and activates more than 30 different enzymes in Ca²⁺-dependent manner. These enzymes are involved in glycogen metabolism, cyclic nucleotide metabolism, secretion, cell motility and protein phosphorylation (reviewed by Lu and Means, 1993). Given the vast number of

enzymes regulated by $\text{Ca}^{2+}/\text{CaM}$ complex it is not surprising that CaM plays a role in controlling cell growth and division.

The role of CaM in cell cycle events is well documented. Intracellular concentrations of calmodulin increase 2-fold at the G_1/S transition and are elevated when cells reenter the cell cycle from G_0 phase (Chafouleas et al., 1982; Chafouleas et al., 1984). Inhibition of CaM by antagonist drugs reversibly blocked cultured cells at the G_1/S boundary and in mitosis (Chafouleas et al., 1982; Sasaki and Hidaka, 1982). Decreasing CaM levels by expression of antisense mRNA caused cell cycle arrest in mouse C127 cells. More detailed analysis revealed that cells are arrested in G_1 , G_2 and metaphase of mitosis. In contrast, overexpression of CaM accelerated progression through G_1/S , G_2/M and shortenes the length of the G_1 phase (Rasmussen and Means, 1989).

Gene disruption experiments performed in *Saccharomyces cerevisiae* (Davis et al., 1986), *Schizosaccharomyces pombe* (Takeda and Yamamoto, 1987) and *Aspergillus nidulans* (Rasmussen et al., 1990) revealed that calmodulin is essential for cell growth. Phenotypic analysis of conditional CaM mutants in *S. cerevisiae* have shown that this protein is required for progression through nuclear division, especially chromosome segregation (Ohya and Anraku, 1989). Site specific mutagenesis of the *S. cerevisiae* CaM gene has demonstrated additional roles in nuclear division, actin localization, cell growth and bud emergence (Ohya and Botstein, 1994). Cell cycle studies using an *A. nidulans* strain conditional for CaM expression indicate that CaM is required at the G_1/S and G_2/M transitions (Rasmussen et al., 1992).

While it is known that CaM is required at multiple cell cycle transitions, the mechanisms involved have not been characterized. Two likely possibilities might be through regulation of other signal transduction pathways such as cAMP-dependent processes or through regulation of protein phosphorylation. Cyclic AMP has been shown to be an important regulator of cell

cycle events. cAMP concentration is elevated during progression through G₁ phase and increases rapidly in proliferating lymphocytes (Whitfield et al., 1980). Addition of cholera toxin to 3T3 cells resulted in increased cAMP levels and stimulated proliferation (Leof et al., 1982). Treatment of 3T3 cells with an inhibitor of cyclic nucleotide phosphodiesterase caused elevation of intracellular cAMP levels and results in a reversible arrest of cells in G₁ phase (Johnson et al., 1988). These results indicate that the elevation and subsequent decrease in cAMP levels are essential for normal progression through the cell cycle. CaM might play a role in this process by activation of the CaM-dependent phosphodiesterase which degrades cAMP, or by activation of adenylyl cyclase, the enzyme responsible for cAMP synthesis (reviewed by Sharma and Wang, 1986). CaM-dependent changes in cAMP concentration might also affect the transcriptional regulation of gene promoters containing cAMP-responsive elements (Roesler et al., 1988).

With regard to reversible protein phosphorylation, CaM can also activate a large number of protein kinases and at least one phosphatase. It is known that modulation of the phosphorylation state of key proteins is essential for cell cycle progression. Consistent with this prediction, studies reveal the importance of multifunctional CaM-dependent kinase II (CaMKII) in cell proliferation. This protein localizes to the interphase nucleus and mitotic apparatus of mammalian cells (Ohta et al., 1990). Investigations in sea urchin eggs suggest a role for CaMKII in nuclear envelope breakdown (Baitinger et al., 1990). Microinjection of a mutant cDNA with constitutive activity initiates maturation of *Xenopus* oocytes (Waldmann et al., 1990), while expression of a truncated, constitutively active form of CaMKII arrested mammalian cells in G₂ phase of the cell cycle (Planas-Silva and Means, 1992). Expression of constitutively active CaMKII in *S. pombe* arrested cells in G₂ and was associated with a failure to activate a key G₂/M regulator - the p34/cdc2 protein kinase (Rasmussen and Rasmussen, 1994).

Studies of CaM-dependent nonmuscle myosin light chain kinase (nmMLCK) also revealed its possible role in regulating cell cycle events. Chicken embryo fibroblast and 3T3 cells incubated with 5' or 3' nmMLCK antisense oligonucleotides proliferated ~2.5-fold slower than control ones (Shoemaker et al., 1990).

Calmodulin also plays a role in regulation of a protein phosphatase known as calcineurin or protein phosphatase 2B. Calcineurin, first detected in skeletal muscle and brain is a serine/threonine phosphatase (Stewart et al., 1982). It is present in numerous mammalian tissues (Klee et al., 1987) and in lower eukaryotic organisms, such as budding yeast (Cyert and Thorner, 1991) and filamentous fungi (Higuchi et al., 1990; Rasmussen et al., 1994). Calcineurin is a heterodimeric protein composed of a calmodulin-binding catalytic subunit (CnA) and a Ca^{2+} -binding regulatory subunit (CnB).

Genes encoding both the CnA and CnB subunits have been isolated from mammals and lower eukaryotes. Two distinct CnA genes have been identified in most mammalian species. Each one gives rise to at least two different mRNAs generated through alternative splicing (Ito et al., 1989; Kuno et al., 1989; Kincaid et al., 1990; Guerini and Klee, 1991). In the yeast *S. cerevisiae*, two CnA genes and one CnB gene have been identified (Cyert et al., 1991; Liu et al., 1991; Cyert and Thorner, 1992). A single CnA gene has been isolated from *A. nidulans* (Rasmussen et al., 1994) and the yeast *S. pombe* (Yoshida et al., 1994, this study).

Recently, important roles for calcineurin in intracellular signal transduction have been discovered as a result of studies examining the effect of immunosuppressant drugs like cyclosporin A or FK-506 on T lymphocyte activation. In the cytoplasm, these drugs bind to intracellular receptors known as cyclophilins or FK-506 binding proteins (reviewed by Clardy, 1995). The drug/receptor complex has been shown to bind and inactivate calcineurin which in turn inhibits expression of the interleukin-2 gene (IL-2). Overexpression of calcineurin can overcome this inhibition (Schreiber and Crabtree, 1992). Recent studies show that calcineurin

dephosphorylates the transcription factor NF κ B, allowing its nuclear translocation and involvement in regulation of the IL-2 gene via another factor, NFAT (McCaffrey et al., 1993). Therefore, in T-cells calcineurin appears to be important in the initiation of proliferation and differentiation by regulating the expression of differentiation specific genes.

Some clues about the role of calcineurin in yeast come from gene disruption experiments. Disruption of one or both CnA genes (*cnal*, *cna2*) in *S. cerevisiae* has no effect on growth rates, heat shock response, sensitivity to nitrogen starvation, mating or sporulation (Nakamura et al., 1992; Cyert et al., 1991). However cells lacking one or both CnA genes lose the ability to recover from growth arrest induced by the a-factor mating pheromone. Overcoming this arrest is equivalent to cell cycle re-entry. Therefore these data show that calcineurin affects yeast proliferation at G₀/G₁ transition positively (Cyert et al., 1991; Cyert and Thorner, 1992). In addition, in *S. cerevisiae* the level of the *cnal* gene product is elevated by a-factor treatment, suggesting involvement of calcineurin in the mating response (Ye and Bretscher, 1991).

Other studies also reveal that calcineurin can negatively affect cell proliferation in yeast. Inappropriate activation of calcineurin in *S. cerevisiae*, attained by increased Ca²⁺ influx or decreased sequestration of Ca²⁺, causes growth inhibition (Cunningham and Fink, 1994). Mutations in the catalytic or regulatory subunits of *S. cerevisiae* calcineurin causes reduced salt tolerance and slow cell growth in alkaline pH medium (Nakamura et al., 1993).

Disruption of the CnA gene in *A. nidulans* is lethal indicating it is an essential gene. CnA mRNA levels in *A. nidulans* vary in a cell cycle-dependent manner, reaching maximum levels after the end of mitosis, likely in G₁ phase (Rasmussen et al., 1994). Together these data suggest that in *A. nidulans* the CnA gene is essential for cell viability and is required for cell cycle events prior to the onset of DNA replication. In *S. pombe* cells lacking a calcineurin

gene are viable but unable to sporulate. Additionally at lower temperatures (e.g. 22°C) cytokinesis is delayed and normal morphogenesis is altered (Yoshida et al., 1994).

Together these data suggest diverse roles for calcineurin in cell functions including cell proliferation, and differentiative processes such as the mating response in yeast or activation of T-cells. In fission yeast vegetatively dividing haploid cells exit the cell cycle at G₁ in response to nutrient deprivation, especially nitrogen starvation, and undergo mating and sporulation (Egel and Egel-Mitani, 1974). Sexual differentiation can be divided in two stages. The first stage involves exit from cell cycle at G₁ phase, induction of mating competency and conjugation of cells of opposite mating type to form a diploid zygote. The second stage which occurs after zygote formation includes pre-meiotic DNA synthesis, meiosis and sporulation. Several changes occur in yeast during mating. These include decreases in the levels of cAMP, changes in protein kinase activity and the expression of mating-specific genes. In addition signal transduction pathways such as the MAP kinase cascade become active (Neiman et al., 1993). Therefore, several potential targets might exist for CnA in yeast.

The goal of this project was to clone the CnA gene of *S. pombe*, characterize CnA mRNA expression during either the cell cycle, or during conditions that trigger the mating response. The results obtained have shown that CnA transcript levels vary during the cell cycle, peaking during the S phase. In addition, its expression is significantly induced by nitrogen deprivation. The observed induction was found to be inhibited by increased intracellular cAMP levels and was dependent on the *ste11* transcription factor. A *ste11* cis-acting TR element was identified in the CnA gene promoter. Together the results suggest that CnA in *S. pombe* is part of gene expression cascade required for mating and sporulation.

Materials and Methods

Strains and Culture Conditions

The following strains of *S. pombe* were used in these studies: wild type, *cdc 25* (*h*⁻, *leu*¹⁻³², *cdc 25-22*) temperature sensitive mutant and *stell*⁻ (*h*⁹⁰, *stell*⁻, *ura4*). All yeast strains were cultured as described (Alfa et al., 1992). *Escherichia coli* strain DH5a was cultured as described previously (Sambrook et al., 1989).

Screening λ Phage Genomic Library

A λ Fix II genomic library was screened as described (Sambrook et al., 1989). Magna Nylon membranes containing λ DNA were hybridized, washed and exposed in the same way as Southern blots.

Isolation of Bacteriophage λ DNA

Host *E. coli* strain JM 109 cells were prepared as described (Sambrook et al., 1989). λ bacteriophage lysates of clones, that were obtained from screening the λ Fix II genomic library were prepared in the following way. A single plaque was picked from a plate, placed in 1 ml of λ diluent (10 mM Tris-HCl, pH 7.5; 10 mM MgSO₄). Phage were allowed to elute for 2 hr. at room temperature, then a 100 μ l aliquot of eluted phage was taken, added to 500 μ l of host bacteria and allowed to adsorb for 30 min at 38°C. To the adsorbed phage was added 37 ml of 2 x YT media (Sambrook et al., 1989) and the sample was then incubated at 38°C for 12-15 hr. to allow infection and subsequent lysis of bacterial cells. Following incubation, the sample was mixed with 100 μ l of chloroform to lyse all remaining bacteria. To the lysate 370 μ l of nuclease buffer was added (5 mg/ml DNase (Sigma), 5 mg/ml of RNase A (Sigma), 50% glycerol, 30 mM sodium acetate, pH 6.8) and the sample incubated at 37°C for 30 min. Sodium chloride was added to a final concentration of 0.96 M and the mixture centrifuged

(5000 x g, 20 min.) to remove bacterial debris. Polyethylene glycol (average mw = 8000) was added to the supernatant (100 mg/ml) and the sample was incubated in ice for 60 min. Precipitated λ phage were collected by centrifugation (5000 x g, 20 min.) then resuspended in 500 μ l of λ diluent buffer. The sample was extracted with an equal volume of chloroform: iso-amyl alcohol (24:1). To the supernatant was added 20 μ l of 0.5 M EDTA, 5 μ l of 20 % SDS, 10 μ l of Proteinase K (Promega) (5 mg/ml) and the sample incubated at 65°C for 30 min. Following incubation, the sample was repeatedly extracted with an equal volume of phenol:chloroform: iso-amyl alcohol 25:24:1 (Sambrook et al., 1989) until no protein remained at the interface. A final extraction with chloroform: iso-amyl alcohol 24:1 was used to remove any remaining traces of phenol. The λ DNA was precipitated by adding 1/10 volume of 3 M NaOAc, pH 5.4, 3 volumes of 95% ethanol and collected by centrifugation (5000 x g, 15 min.). The pellet was washed once with 70% ethanol and dried under vacuum. The DNA pellet was resuspended in 500 μ l of TE buffer (10 mM Tris-HCl, 1 mM EDTA) and quantified by A_{260} . The typical yield was 50-100 μ g DNA per lysate.

Total RNA isolation

Total RNA was isolated by one of two methods: extraction with hot acid phenol (Ausubel et al., 1991) or by using glass beads to break cells, followed by extractions with phenol (Moreno et al., 1991). Following isolation RNA was resuspended in sterile water and quantified by A_{260} .

Genomic DNA isolation

S. pombe cultures containing 3 - 5 x 10⁷ cells per ml were used for each isolation. Genomic DNA was isolated as previously described (Alfa et al., 1992).

Plasmid and Cosmid Isolation

Small-scale preparation of plasmid and cosmid DNA was performed by the alkaline lysis method as previously described (Sambrook et al., 1989). For large-scale preparation alkaline lysis method and precipitation with PEG/NaCl was used as described (Sambrook et al., 1989). Following isolation DNA was resuspended in TE buffer and quantified by A_{260} .

Synthesis of Radiolabelled Hybridization Probes

Plasmid DNA was digested with the appropriate restriction enzyme (Promega) and fragments resolved by low melting point agarose electrophoresis as described (Sambrook et al., 1989). The desired fragment was cut out from the gel, incubated at 70°C for 10 min. and purified using Magic DNA Clean up resin (Promega). For each labeling reaction 200 ng of DNA was labeled with a ^{32}P -dCTP (3000 Ci/mM, Amersham) by the random primer method (Sambrook et al., 1989) using random hexanucleotides (Pharmacia). Labeled DNA was separated from unincorporated nucleotides by G-50 Sephadex chromatography. After labeling, incorporation was quantified by liquid scintillation counting.

Southern Blot Analysis

For analysis of isolated DNA by the method of Southern (1975), the following procedure was used. Isolated DNA was digested with appropriate restriction enzyme and fragments resolved by agarose gel electrophoresis (Sambrook et al., 1989). Following electrophoresis the gel was soaked in 500 ml of denaturation solution (1.5 M NaCl; 0.5 N NaOH) for 45 min, followed by a 10 min wash in milli-Q water, and 30 min incubation in neutralization solution (1.5 M NaCl, 1 M Tris-HCl pH 8.0). Then DNA was transferred to Magna Nylon transfer membrane by capillary blotting overnight using 20 x SSC (3 M NaCl, 0.3 M sodium citrate pH 7.0) as the transfer buffer. After transfer, filters were air dried for 30 min. and the DNA cross-linked to the membrane using a UV Stratacross-linker 2400 (1200 Joules/cm²).

Northern Blot and Slot Blot Analysis of Total RNA

Equal amounts of total RNA in sterile water were dried under vacuum in a sterile microfuge tube. The lyophilized RNA was resuspended in 5 μ l sterile water and 15 μ l denaturing sample buffer (65% de-ionized formamide; 8% formaldehyde; 2.6x Borate buffer where 20 x Borate buffer = 400 mM Boric Acid, 4 mM EDTA pH 8.3) and samples incubated in ice for 15 min. Prior to electrophoresis RNA was denatured by heating at 65°C for 5 min followed by rapid chilling in ice. Three μ l of loading buffer (0.1% bromophenol blue; 0.1% xylene cyanol; 0.1 M EDTA) was added and samples loaded onto a denaturing agarose gel (1% agarose; 3% formaldehyde; 2.6x borate buffer). Electrophoresis was carried out at 75 volts until the bromophenol blue marker dye reached the end of the gel. Following electrophoresis the gel was stained in water containing 1 μ g/ml ethidium bromide to visualize RNA and ensure equivalent loading. RNA was then transferred to Magna Nylon transfer membrane using the same procedure as described for Southern blots. After overnight transfer filters were air dried for 30 min and cross-linked in UV Stratacross-linker 2400 (1200 Joules/cm²).

For slot-blot analysis, 10 μ g of RNA in sterile water was mixed with an equal volume of SSCF (3 vol 20 x SSC, 2 vol 37% formaldehyde) and denatured at 68°C for 15 min. The sample was chilled in ice and an equal volume of 15 x SSC added. Blotting onto Magna Nylon membrane was performed according to manufacturer's specifications using a Mini-fold slot blot apparatus (Tyler Research).

Hybridization Conditions

Following cross-linking, filters were pre-hybridized for 2 hr at 42°C in 50% de-ionized formamide, 5 x Denhardt's, 6 x SSC, 0.5% SDS, 100 μ g/ml denatured herring sperm DNA solution (Sambrook et al., 1989). Hybridization was carried out at the same temperature for 18 hr in a fresh solution of the same composition as that used for pre-hybridization. Probe concentration was 5 x 10⁶ cpm/ml. After hybridization filters were washed 5 times in 2 x

SSC, 0.1% SDS at room temperature, and twice for 15 min in 0.1 x SSC, 0.1% SDS at 50-55°C. After washing filters were covered with Saran Wrap and exposed to Kodak X-OMAT AR at -70°C using intensifying screens.

Subcloning

The plasmid pPGEM3Zf(-) was used as a cloning vector. Vector DNA was digested with the appropriate restriction enzyme, then precipitated with 3 volumes of 95% ethanol and 1/10 volume of 3 M sodium acetate, pH 5.2. The pellet was collected by centrifugation (14,000 x g; 10 min), washed with 70% ethanol, and dried under the vacuum for 5 min. The DNA was then resuspended in 1x CIP buffer (0.05 M Tris-HCl, pH 9.0, 1mM MgCl₂, 0.1 mM ZnCl₂, 1 mM spermidine) and dephosphorylated with 10 U of calf intestinal alkaline phosphatase enzyme (Promega) at 37°C for 1 hr. The vector DNA was then extracted with equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1), ethanol precipitated, washed in 70% ethanol and dried under the vacuum as described above and resuspended in 40 ul of TE buffer.

Insert DNA was prepared as follows. Plasmid or cosmid DNA was digested with the appropriate restriction enzyme and fragments resolved by electrophoresis in low melting point agarose. The desired insert DNA was cut from the gel, incubated for 10 min at 70°C to melt the rest of the gel and DNA was purified using a Magic DNA Clean up kit (Promega). Aliquots of insert and vector DNA samples were analyzed by agarose gel electrophoresis to estimate the amount of DNA in the sample.

Ligation reactions were performed in a 20 ul volume (50 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 1mM ATP, 1mM DTT, 5% polyethylene glycol-8000) with 1U of T4 DNA ligase (Promega) at room temperature overnight. Fresh competent *E. coli* DH5α cells were prepared as described (Hanahan, 1983). Ligated DNA was transformed into *E. coli* as described (Sambrook et al., 1989) and ampicillin resistant colonies selected for further analysis.

DNA Sequencing

DNA sequencing was performed using the dideoxy-mediated chain termination method (Sambrook et al., 1989) and a Sequenase version 2.0 kit (United States Biochemical). Samples were loaded on 6% polyacrylamide gel using wedge spacers to improve resolution. Following electrophoresis the gel was fixed for 10 min in 10% methanol, 10% glacial acetic acid and dried under the vacuum at 80°C for 45 min. Dried gel was exposed to Kodak X-OMAT AR film at room temperature overnight. Exonuclease III digestion (Henikoff, 1984) was used to create uni-directional deletions for sequence analysis.

Synchronization of S. pombe.

For experiments requiring synchronization samples of a temperature sensitive (*cdc 25-22*, *leu 1-32 ts*) *S. pombe* strain were used. *cdc 25-22* can proliferate at the permissive temperature (25°C) but reversibly arrests at the G₂/M boundary of the cell cycle at the restrictive temperature (36°C). To obtain synchronous cultures, cells were first grown at the permissive temperature until they reached density 5 x 10⁶ cells/ml. To induce synchrony, the culture was shifted to the restrictive temperature for 4.25 hr. To release cells from the G₂ arrest, the culture was returned to permissive conditions and samples taken at 20 min intervals. To examine nuclear morphology, cells were fixed in 3.7% formaldehyde and stained with 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) as described (Moreno et al., 1991). For RNA isolation 5 x 10⁸ cells were washed with sterile water, then frozen in liquid nitrogen. RNA was isolated and analyzed as described above.

Nutritional Shifts

A wild type *S. pombe* strain was used for these experiments. Log phase cultures were shifted to the following nutritional conditions: a) nitrogen-free medium, b) glucose-free

medium, c) regular minimal medium, as described (Alfa et al., 1992). Cells were cultured until growth arrested then samples collected and RNA isolated to examine CnA mRNA levels.

Nitrogen Starvation Time Courses

Exponentially growing cultures of *S. pombe* were washed, then shifted to nitrogen free minimal media as described (Alfa et al., 1992) and cells harvested for RNA isolation at various time points.

Results

The amino acid sequences of CnA genes from 4 different species (*A. nidulans*, *N. crassa*, *H. sapiens*, *S. cerevisiae* CnA1) were compared and conserved regions present in all genes were chosen to construct primers for PCR synthesis of a *S. pombe* CnA probe (Fig. 1). The region selected lies between amino acid 146 (*A. nidulans*) or 177 (*S. cerevisiae* CnA1) up to amino acids 344 (*A. nidulans*) or 382 (*S. cerevisiae* CnA1) encompassing a region predicted to encode approximately 200 amino acids (Fig. 2A). Oligonucleotides directed towards the 5' and 3' ends of the region were obtained from Dr. Randall Kincaid, Immunology Section NIAAA and used as primers in PCR reactions with *S. pombe* genomic DNA as the template (Fig. 1). A 500 bp PCR product was subcloned into the plasmid vector pGEM3Zf(-) and sequenced. The predicted protein sequence encoded by the PCR product was compared with other CnA homologues (Fig. 3). Sequences were aligned using a sequence analysis software package (Genetics Computer Group). The peptide sequence encoded by the PCR-derived CnA probe had 80% identity to the corresponding region of the *A. nidulans* CnA gene product and 70% to the *H. sapiens* CnA. This confirms that the PCR product corresponds to a portion of the *S. pombe* CnA gene.

In order to isolate and clone the entire *S. pombe* CnA gene, λ Fix II and ordered P₁ bacteriophage *S. pombe* genomic libraries were each screened with the CnA PCR probe. Primary screening of 1 Fix II library identified two clones A₁ and A₂ as containing the *S. pombe* CnA gene (Fig. 4). A₁ was plaque purified after secondary and tertiary screening. From the P₁ library twelve clones were identified as containing the CnA gene (Fig. 5). Based on the P₁ library screening results, and according to the known location of these clones on the ordered P₁ map, the chromosomal locus of CnA gene lies between the *cdc10* and *top1* genes on chromosome II (Fig. 7C). Computer analysis of the P₁ library hybridization results by Dr.

Elmar Meier (ICRF, London UK) identified clone 32H9 in an ordered *S. pombe* genomic cosmid library as containing the CnA gene.

To confirm that cosmid 32H9 did contain the CnA gene, genomic and cosmid 32H9 DNA were analyzed by Southern blot hybridization to the CnA PCR probe. Hybridizing bands corresponding to the 2.1 kb EcoRI and two adjacent 5.1 kb, 1.6 kb HindIII fragments were identical in both DNA sources (Fig. 6). This confirms that cosmid 32H9 does contain the *S. pombe* CnA gene. Based on southern blot results a partial restriction map of the *S. pombe* CnA gene was constructed (Fig. 7A). The 2.1 kb EcoRI fragment and 5.1 and 1.6 kb HindIII fragments were subcloned into the vector pGEM3Zf(-) and sequenced (Fig. 8).

The sequencing strategy used is shown in Fig. 7A. While these studies were in progress another group reported cloning a CnA homologue from *S. pombe* (Yoshida et al., 1994). Comparison of our sequence with theirs, indicated that we had cloned identical genes. The putative CnA gene product consists of 554 amino acids. Alignment the protein sequence with rat, human and *S. cerevisiae* calcineurin homologues revealed extensive homology to these proteins (Fig. 9). The highest identity was observed between *S. pombe* CnA and rat CnA2 (57%), human CnA2 (51%) and *S. cerevisiae* CnA1 (45%). Within the conserved region of the protein, spanning residues 108-407, identity increased to 79, 79 and 64% respectively (Kuno et al., 1989; Guerini and Klee, 1989; Cyert et al., 1991).

Because CaM-dependent processes have been shown to be required for cell cycle progression (reviewed by Lu and Means, 1993), CnA mRNA expression was examined during the cell cycle. Cultures of exponentially growing *cdc25-22* cells were incubated at the restrictive temperature (36°C) for 4.25 hrs to arrest cells at G₂/M boundary. Arrested cells were shifted to permissive conditions to permit synchronous progression, and samples removed for RNA isolation. The degree of synchrony was monitored by microscopic observation of nuclear morphology and percentage of binucleated cells following DAPI

staining. Calcineurin mRNA levels were determined by Northern blot analysis (Fig. 10). Under these conditions cells passed through two synchronous cell cycles. The first mitotic division was completed within 100 min and a second within 200 min after shifting to permissive conditions. Calcineurin A mRNA levels varied in cell cycle dependent manner reaching two peaks of expression at 100 and 220 minutes after release. Based on cytological criteria (% of binucleated cells) the results indicate that CnA mRNA levels increase after segregation of nuclei, indicating that the peak of expression occurs coincident with S phase (Fig. 10).

Calcineurin A mRNA levels were also examined under various nutritional conditions. Washed exponentially growing wild-type *S. pombe* cells were shifted either to nitrogen limiting medium, glucose limiting medium or allowed to grow in complete medium to stationary phase. After 18 hrs cells were collected for total RNA isolation. As a positive control, total RNA from exponentially growing cells was also examined. mRNA levels for both histone H2A, a control of entry into S-phase (Matsumoto and Yanagida, 1985), and CnA were determined by slot blot hybridization (Fig. 12). As shown, both histone H2A and CnA mRNA levels varied in different nutritional conditions. The histone H2A transcript was lowest when glucose was limited, compared to slightly higher expression in nitrogen limiting and stationary phase conditions. However while CnA mRNA levels were comparatively low in glucose limiting conditions, they were significantly increased in nitrogen limiting medium or after growth to stationary phase. To better estimate the differences, serial dilution of RNA samples was performed starting with equal amounts of total RNA (10 ug) and analyzed by slot-blot hybridization (Fig. 11, 12). The results show that the levels of CnA mRNA in cells starved for nitrogen are more than 8-fold higher than in exponentially growing cells. The levels of expression in cells grown to stationary phase are at least 4-fold higher than is observed in exponentially growing cells (Fig. 11). The amounts of histone H2A mRNA

remained the same indicating that CnA mRNA levels are selectively induced during these changes in nutritional status (Fig. 12).

The time course of nitrogen starvation induction of CnA mRNA expression was also examined by slot-blot hybridization. The results show that increased levels of CnA mRNA can be detected within 2 hr following the shift to nitrogen limiting medium (Fig. 13A). Maximal levels were detected within 8 hr and remained at peak levels for at least 25 hr.

As described above the mating process in *S. pombe* is induced by nitrogen starvation and mating pheromones (Egel and Egel-Matani, 1974; Issiki et al., 1992), and a few genes that regulate gene expression during meiosis have been characterized. For example it is known that the protein kinase *pat1/ran1* inhibits the mating response in *S. pombe* by inhibiting *mei2* gene function, which is essential for meiosis (Watanabe et al., 1988). In addition upon nitrogen starvation, levels of intracellular cAMP are reduced by 50% (Fukui et al., 1986; Mochizuki and Yamamoto, 1992). This reduction inactivates the cAMP-dependent protein kinase, and allows expression of the *ste11* gene. *ste11* encodes a transcription factor responsible for activation of some genes involved in mating and meiosis. It is known that the *ste11* gene product regulates the expression of genes via a 10 bp cis-acting (TTCITTGTTY) TR-element present in the 5' untranslated region of responsive genes (Sugimoto et al., 1991). Based on that knowledge, the *S. pombe* CnA nucleotide sequence was analyzed to search for this regulatory element. A putative element matching at 9/10 positions (TTCITTGCTT) was identified in the promoter of CnA gene starting at -137 nucleotides relative to the initiation methionine codon.

To determine if nitrogen starvation triggers high expression of mRNA CnA via *ste11* the following experiments were performed. Washed exponentially growing *S. pombe* cells were starved for nitrogen in medium with or without 5 mM caffeine, 10 mM cAMP. The presence of these compounds increases the intracellular levels of cAMP and in turn activates cAMP-

dependent protein kinase. Activation of this enzyme inhibits *ste11* expression and therefore any genes regulated by *ste11* (Watanabe et al., 1988; Sugimoto et al., 1991). Cells were allowed to grow for 18 hr then collected for RNA isolation. As a control, total RNA was isolated from exponentially growing *S. pombe* cells grown with or without caffeine and cAMP. mRNA levels were examined by slot blot hybridization (Fig. 13B). As we had observed earlier CnA mRNA levels in the untreated cells increased upon nitrogen deprivation. In contrast the presence of extracellular caffeine and cAMP inhibited this induction. These results indicate that in *S. pombe* nitrogen starvation triggers the expression of CnA mRNA through *ste11*. To test this directly the levels of CnA mRNA during nitrogen starvation were examined in normal cells and in a strain lacking *ste11* gene. The levels of both histone H2A and CnA mRNA were determined by slot blot hybridization (Fig.13C). As can be seen the expression of both histone H2A and CnA mRNA remain uninduced in the *ste11*⁻ strain. The increase in CnA mRNA level seen during nitrogen starvation of wild type *S. pombe* is not observed in strain lacking functional *ste11* gene. The results indicate that in *S. pombe* the *ste11* transcription factor is an upstream regulator of CnA gene expression during nitrogen starvation.

Discussion

Using a PCR-derived genomic fragment of the *S. pombe* CnA gene as a probe, the entire *S. pombe* CnA gene was isolated from λ phage and cosmid libraries. Based on computer analysis of hybridization of the probe to an ordered P₁ phage library the CnA gene was mapped to chromosome II of *S. pombe* between the *cdc10* and *top1* genes. Sequence analysis shows the CnA gene is 1865 bp in length and has two introns 45 and 155 bp long (Fig. 7; Fig. 8). The gene encodes a putative product of 554 amino acids. The CnA mRNA is expressed in cell cycle dependent manner with transcript levels maximal in S phase. In addition expression of the CnA gene was shown to be induced by nitrogen deprivation. This induction was shown to be inhibited by increased cAMP levels and dependent on the *ste11* transcription factor. A TR element was identified in CnA gene promoter at the position -137 relative to the translation initiation codon (Fig. 8).

Recently, while this study was in progress another group also cloned the CnA gene from *S. pombe*, which they named *ppb1*⁺ (Yoshida et al., 1994). The gene was screened from a different *S. pombe* cosmid genomic library (Mizukami et al., 1993). While the sequences of our clone and theirs are identical, their mapping studies place the *ppb1*⁺ gene on chromosome I while our studies indicate it is on chromosome II. Our mapping results were provided by Dr. Elmar Maier (ICRF, London) who sent a cosmid (32H9) from which we subcloned the *S. pombe* CnA gene. 32H9 maps to chromosome II (Hoheisel et al., 1993) and so the only conclusion we can make is that one of the libraries has been ordered incorrectly. One way to resolve this question would be to generate conditional CnA mutants and then use standard genetic analysis relative to map the *ppb1*⁺ gene relative to known markers.

Sequence analysis of the *S. pombe* CnA gene reveals high homology to CnA genes from other species. The predicted *S. pombe* protein has 57% identity to rat CnA2 homologue (Kuno et al., 1989), 51% to human CnA2 (Guerini and Klee, 1991) and 45% to budding yeast

CnA1 (Cyert et al., 1991). Within the conserved region of the protein, spanning residues 108-407, identity increases to 79, 79 and 64% respectively. These findings confirm previous results showing that phosphoprotein phosphatases are among the most highly conserved enzymes known (reviewed by Cyert et al., 1991). Calcineurin A homologues from different species show high degree of identity within this conserved region differing mostly at the N and C termini (Cyert et al., 1991; Rasmussen et al., 1994; Yoshida et al., 1994).

Low-stringency hybridization experiments suggest the *S. pombe* CnA gene is unique (Yoshida et al., 1994). In our studies a single product is always obtained by PCR using degenerate oligonucleotides and *S. pombe* genomic DNA as a template, consistent with this interpretation.

Gene disruption reveals that the CnA gene in *S. pombe* is not strictly essential for exponential growth at regular temperature (33°C) conditions, however cytokinesis and cell polarity control are slightly affected. Reduction of the temperature to 22°C increases this irregularity (Yoshida et al., 1994). CnA gene disruption experiments in budding yeast have shown that cells lacking both the *CnA1* and *CnA2* genes are still viable (Cyert et al., 1991). These findings suggest that for vegetative growth CnA is either dispensable, or its function can be complemented by other enzymes. These could potentially be other phosphoprotein phosphatases, since the catalytic domains of PP1, PP2A and PP2B phosphatases are homologues to each other (reviewed by Nakamura et al., 1992). Another possibility is that in both species there are homologues of calcineurin not detected even by low stringency hybridization. These findings are in contrast with the results obtained in *A. nidulans*, where disruption of the only identified CnA gene was shown to be lethal. In this organism loss of the CnA gene arrested cell proliferation in the G₁ phase of the cell cycle (Rasmussen et al., 1994).

Calcineurin A mRNA expression in *S. pombe* was found to be regulated in cell cycle dependent manner. Transcript levels are maximal in S phase. The differences in its expression

during other phases of the cell cycle and S phase are not very great (approximately 2-fold). However, these results are comparable to studies performed in *A. nidulans*, where CnA mRNA levels during synchronous cell cycle progression reach a peak of expression in G₁ phase.

The role for CnA in G₁/S progression in *A. nidulans* is clear, since loss of the gene arrests cells in G₁ phase (Rasmussen et al., 1994). However the mechanism of action and possible substrates of calcineurin in *A. nidulans* are unknown. Calcineurin A gene disruption experiments in *S. pombe* and *S. cerevisiae* do not suggest a function during G₁ phase. Overexpression of a CnA cDNA in *S. pombe* causes abnormalities in cell shape and nuclear or spindle body positioning (Yoshida et al., 1994). It is possible that in the previously mentioned study, high levels of CnA expression obtained by using the wild-type version of the thiamine-regulated *nmt1* gene promoter inserted in the plasmid pREP1 may have had non-specific effects. Unfortunately nothing is known about CnA mRNA expression during the cell cycle in other fungal species such as *Neurospora crassa* or *S. cerevisiae*, where homologues have been cloned. It is known that gene expression is additionally regulated in the cytoplasm by the level of mRNA stability and mRNA translation. Activity of the synthesized enzyme also depends on intracellular conditions, in the case of calcineurin the presence of Ca²⁺ and calmodulin. Therefore the levels of mRNA expression of the gene may not reflect the amount of an active enzyme in the cell.

Experiments in *S. cerevisiae* strongly point to the role of calcineurin during sexual differentiation (Cyert et al., 1991; Yoshida et al., 1994). The present study shows that nitrogen deprivation, which in *S. pombe* is a signal for cells to undergo mating, meiosis and sporulation, induces CnA mRNA expression. The expression of genes like *mei2*, *ste4*, *ste11* or *repl* which are involved in *S. pombe* sexual differentiation, has been shown to be induced by nitrogen deprivation (Sugimoto et al., 1991; Okazaki et al., 1991). In this species starvation causes inactivation of cAMP-dependent protein kinase, resulting in induction of

stell gene, whose product is a transcription factor that activates a subset of genes essential in sexual differentiation (Watanabe et al., 1988; Sugimoto et al., 1991). We found CnA mRNA expression was inhibited by elevation of intracellular cAMP levels. In addition nitrogen starvation dependent increased mRNA levels were abolished in *S. pombe* strain lacking functional *stell* gene. A search of the CnA gene promoter revealed the presence of a potential *stell* binding site (TTCTTTGTTY) at the position -137 relative to the translation initiation codon.

The induction of CnA mRNA expression during nitrogen deprivation increased within 2 hr following the shift to nitrogen limiting medium, reached its peak after 8 hr and remained elevated for 25 hrs. Not all genes activated by nitrogen deprivation respond identically. For instance, mRNA levels of the *res2* gene, achieve maximum expression within 6 hrs, but return to initial levels after 12 hr (Miyamoto et al., 1994). The pattern of CnA mRNA expression during nitrogen starvation suggests that calcineurin may be required at the initiation of mating and also during meiosis and sporulation. Consistent with these results, it has been shown that a loss of calcineurin function causes cells to be unable to form spores (Yoshida et al., 1994). Taken together it seems that in *S. pombe* this enzyme plays a crucial role in the mating response, when upon low nutrient levels a decision is made to exit the mitotic cell cycle and undergo mating, meiosis and sporulation, while in budding yeast and *A. nidulans* calcineurin is required during the G₀/G₁ transition (Cyert et al., 1991, Rasmussen et al., 1994). This might be due to distinct sexual differentiation patterns in fission and budding yeast. In *S. pombe*, typically proliferating at the haploid stage, mating and conjugation is stimulated by low nutrient levels especially nitrogen deprivation (Alfa et al., 1992). In contrast, *S. cerevisiae* conjugates under favorable growth conditions and diploid cells proliferate asexually by budding, until low nutrient levels induce meiosis (Thorner et al., 1981). That may be why it is difficult to compare gene expression pathways leading to mating and sporulation in both

species. In addition CnA mRNA expression during sexual differentiation in *S. cerevisiae* has not been examined yet.

The mechanisms of calcineurin action in *S. pombe* during mating and sporulation may be comparable to those that function during activation of T-cells. Calcineurin was shown to dephosphorylate the cytoplasm NF-kB transcription factor, allowing it to translocate to the nucleus and activate IL-2 gene expression (McCaffrey et al., 1993). It is possible that in fission yeast calcineurin dephosphorylates specific transcription factors, which regulate genes essential for mating and sporulation. It is also possible that this phosphatase antagonizes protein kinases known to inhibit sexual differentiation. Two such kinases have been characterized in *S. pombe*. One of them is cAMP-dependent protein kinase, whose activity prevents expression of *ste11* gene, in turn prevents expression of *ste11*-dependent genes. Another the *pat1/ran1* kinase which inhibits the activity of the *mei2* gene (reviewed Sugiyama et al., 1993). At present little is known about calcineurin substrates or their functions. Further research will need to concentrate on identifying specific substrates for this enzyme and their role downstream of calcineurin in the mating response.

Figure 1. Sequence of PCR Primers Con S-1 and Con AS-1.

Primers Con S-1 and Con AS-1 were used for PCR amplification of a segment of the *S. pombe* CnA gene. These degenerate oligonucleotides were synthesized based on two highly conserved regions of known CnA proteins. They were provided by Dr. Randall Kincaid (NIH).

Con S-1

5'	G(A/G)	GG(X)	AA(T/C)	CA(T/C)	GA(A/G)	TG
	R	G	N	H	E	C

Con AS-1

5'	CCA	(A/G)GT	GAA	(A/T)AC	(A/G)TC	CAT
anti	M	D	V	F	T	W

Figure 2. Construction of the PCR Probe.

(A). Location of the primers Con S-1 and Con AS-1 relative to the predicted amino acid sequence of a highly homologous region of CnA genes from 4 different species is shown. The numbering indicates the range of amino acids within the protein sequence, and the length of the region. The primers were used along with *S. pombe* genomic DNA as the template.

(B). The box illustrates the entire CnA gene. The shaded region, is the region repositential by the PCR probe. Positions of the primers are indicated by arrows. The expected length of PCR product is indicated below.

A.

<i>A. nidulans</i>	146	RGNHEC.....198.aa.....MDVFTW	344
<i>N. crassa</i>	146	RGNHEC.....198.aa.....MDVFTW	344
<i>H. sapiens</i>	175	RGNHEC.....198.aa.....MDVFTW	373
<i>S.c (CNA1)</i>	177	RGNHEC.....205.aa.....MDVFTW	382

B.

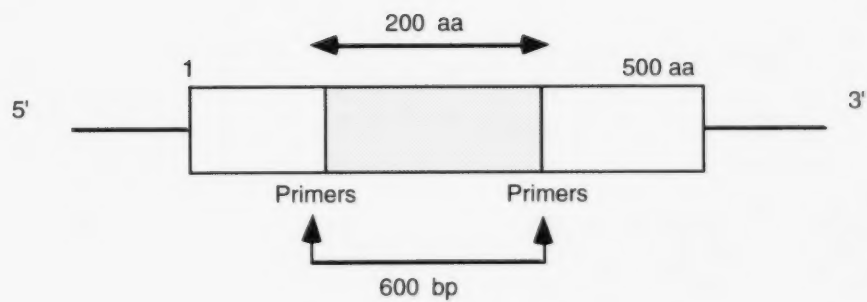


Figure 3. Alignment of the Predicted Amino Acid Sequence of the *S. pombe* CnA PCR Product with Other CnA Homologues.

Alignment of the predicted amino acid sequence of the CnA PCR product with other CnA homologues. Sequences were aligned using the Sequence Analysis Software Package from Genetics Computer Group (GCG). Amino acid residues are displayed in one letter notation and are numbered at the beginning of each line. Identical amino acids are indicated by "-". The results show that the PCR probe encodes a portion of the *S. pombe* CnA gene. The "?" denotes residue which could not be assigned due to errors in the initial sequencing the PCR probe.

S. pombe	177	GNHECAHLTDYFTFKLECTHKYN??VYEAC
A. nidulans	146	-----R-----K---SERI----
H. sapiens	176	-----R---E-----Q--KI--SER-----
S. pombe	207	LQSF?CLPLAAIMNKQFLCVHGGLSPELHT
A. nidulans	176	I---CA-----V-----I-----
H. sapiens	206	MEA-DS-----LI-Q-----I--
S. pombe	237	LNDIRMINRFCEPPTHGLMCDLLWSDPLED
A. nidulans	206	-E--KS-D--R-----I--A-----
H. sapiens	236	-D---R-D--R----Q-----I--A--S--
S. pombe	267	FGSEKSNKHFIHNNVR
A. nidulans	236	--Q---TG DY-----S--
H. sapiens	266	--N---GE--SK-T--

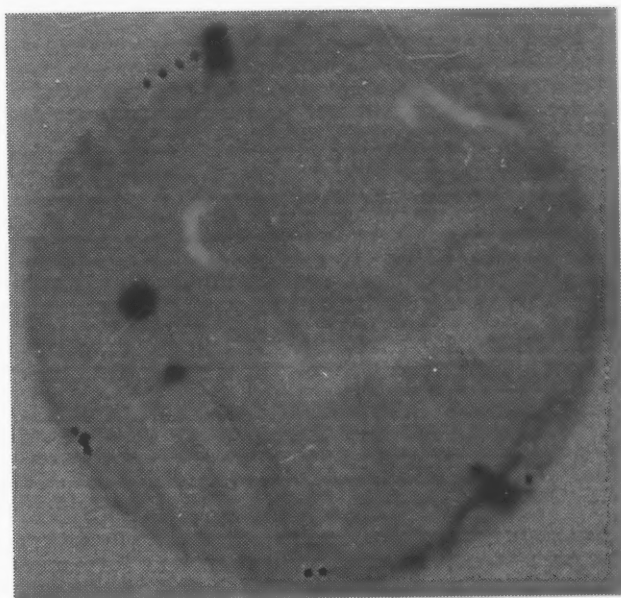
Figure 4. Screening of λ Bacteriophage Fix II *S. pombe* Genomic Library with the PCR probe.

A λ bacteriophage *S. pombe* genomic library was screened using the PCR probe as described in Materials and Methods.

(A) Autoradiogram of the primary screening.

(B) Tertiary screen of the plaque-purified phage.

A.



B.

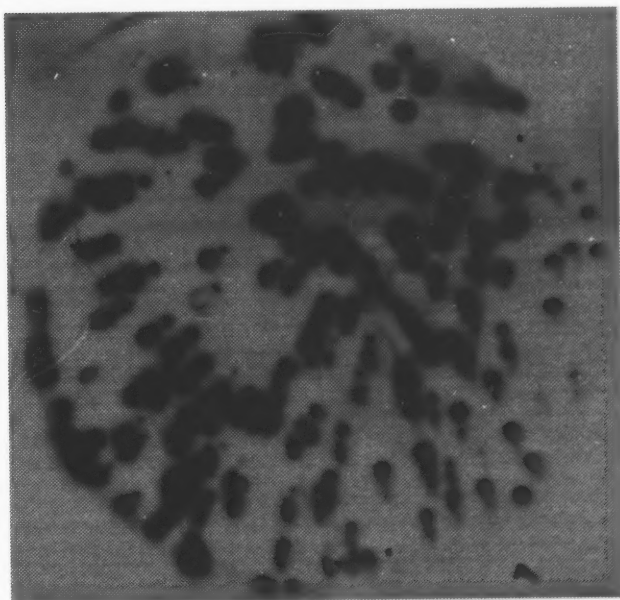


Figure 5. Screening of P₁ *S. pombe* Genomic Library with the PCR probe.

An ordered P₁ bacteriophage library was screened using the PCR probe as described in Materials and Methods.

(A). Diagram representing the location of the clones on the filter.

(B). Autoradiogram of the filter hybridized to the PCR probe. Exposure time was 40 min. Twelve clones from the library were identified as appearing to contain the *S. pombe* CnA gene.

[illegible]

Figure 6. Southern Blot Analysis of *S. pombe* Genomic and Cosmid 32H9 DNA.

S. pombe genomic (gen) and cosmid (cos) 32H9 DNA were digested with EcoRI (E) or HindIII (H), separated on 1% agarose gels, blotted onto Magna Nylon Membrane and hybridized to the PCR probe, as described in Materials and Methods. The sizes of fragments in kb are indicated for each panel.

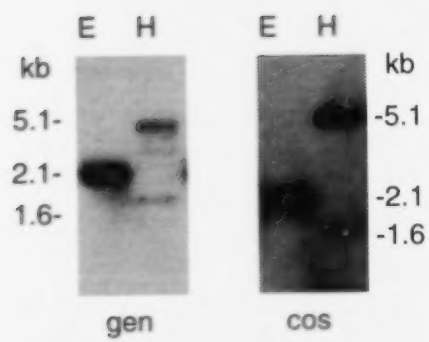


Figure 7. Organization of the *S. pombe* CnA Gene.

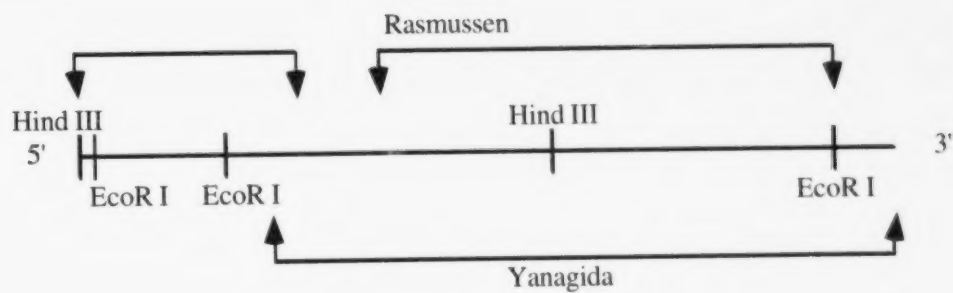
The organization of the *S. pombe* CnA gene is shown.

(A). Partial restriction map showing positions of EcoRI and HindIII sites. The top line (labeled Rasmussen) indicates sequence obtained in this study, while the bottom line (labeled Yanagida) represents sequence published while this study was in progress (Yoshida et al., 1994).

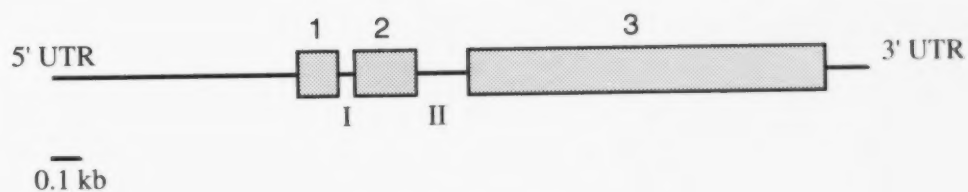
(B). Intron/exon locations for the *S. pombe* CnA gene. Exons are indicated by shaded area and numbered 1, 2 and 3. Introns are indicated by Roman numerals. Intron positions were identified by Yoshida et al. (1994).

(C). Location of the calcineurin A gene on *S. pombe* chromosome II.

A.



B.



C.

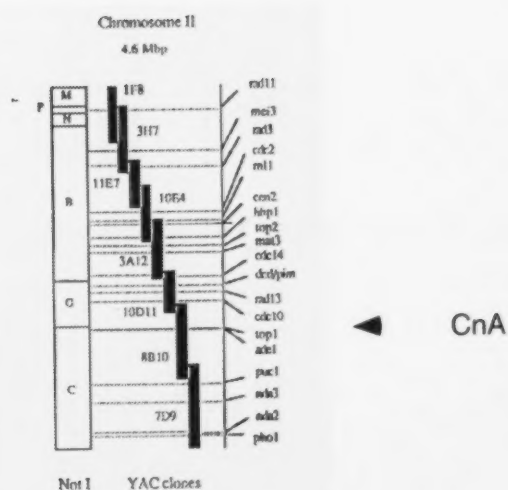


Figure 8. Nucleotide and Deduced Amino Acid Sequence of the *S. pombe* CnA gene.

The complete nucleotide sequence of the *S. pombe* CnA gene is shown including 867 non-transcribed nucleotides of 5' flanking region. Protein coding regions are shown in upper case letters, while non-translated regions in lower case letters. Nucleotide numbering is relative to the initiation codon of the predicted protein coding sequence. The position of the TR-element (nucleotides -137 to -128) is underlined.

-867 gcttttttgtgtgcttttgggaattctctttcttagaagtggtccaatgtcctattgagag
-808 cataaaagtttgtaagatatgggtaattttttacatttcttgcattattcatttctttc
-749 ctttggcttatcccataaattttacatatgttttcgtttttgccatatatcttcacactt
-690 gcaacactctttttctattttcctttacttctataaaaaatacccaaatttacatttatttt
-631 gcttattcttttctaaaaatcggtcttttgtctaatctttactacggaaagcgatgagaa
-572 atcaaagaatattttacaaatctctaaatttgacgtagccgtctaaacaaaaataaaaag
-513 attattaactcttaaaagtgttttatgggatatttattttatcaataaaactatcggttatt
-454 tcagttaggtaatcatggaatgtaagtttcatcaaatcaacttagtttgcttctctcaa
-395 aaatgagcaaggcaatataattaggaaggaattcaggtagtagtttggttccctttctac
-336 aagttcatttcttattactaacacgattgggttattaagatatgtttgtctaaagcggcga
-277 ttgtttgtaaatttttaaatatacagaagtaaagattatgatgaaatatatctatatta
-218 taaaaaaaaaaaaaagtacttagaattgtaaacagatatgttttttgttcacatgggttc
-159 attgagaaaagcaattacgaatttcttttgcgttttcgttaaaccattttccaaaaccgcc
-100 gggacgatgtaagtataacaaaccagaatctacaaaactttttgcaagaatatctagc
-41 attttaccaggctgtatataggttgcaaaagttactaat ATG ACT TCG GGT
Met Thr Ser Gly
+13 CCT CAT AAT TTA GAA GAC CCA ACA GTT CGA GCA ATT CGC CAA AAA
Pro His Asn Leu Glu Asp Pro Thr Val Arg Ala Ile Arg Gln Lys
+58 AAT CAA GCT CCT TCT CAT GAT TTT ACA ATA TTT GTT CAA GAA GAT
Asn Gln Ala Pro Ser His Asp Phe Thr Ile Phe Val Gln Glu Asp
+103 GGC TCT AGT GTT AGC ACA CTG GAT AGA GTA GTG AAA AGT acggata
Gly Cys Arg Val Ser Thr Leu Asp Arg Val Val Lys Ser
+149 ttttttgaaagaaattagtccttattaacaatcaaagat GTT CAA GCA CCA GCA
Val Gln Ala Pro Ala
+202 ACT TAT ATT CCG ACG GAC GTG GAG TTT TTT GAT ATT AAT GAA CCT
Thr Tyr Ile Pro Thr Asp Val Glu Phe Phe Asp Ile Asn Glu Pro
+247 GAT AAA CCT GAC TTG CAT TTT CTT CGC AAT CAT TTC ATT CGG GAA
Asp Lys Pro Asp Leu His Phe Leu Arg Asn His Phe Ile Arg Glu
+292 GGC CGT TTA TCT GAG GAA CAA ACG TTG TAT ATT ATA AAA AAG GCT
Gly Arg Leu Ser Glu Glu Gln Thr Leu Tyr Ile Ile Lys Lys Ala
+337 ACA GAA ATT TTG AAG TCA GAA GAC AAC TTG ATT GAG GTG GAT GCA
Thr Glu Ile Leu Lys Ser Glu Asp Asn Leu Ile Glu Val Asp Ala

+382 CCT GTC ACT GGT atgttgattatcacctttcatatttcggccctaattactataa
 Pro Val Thr Gly

+437 ggtaaaatattagaactttgaaatgtaatttatgactatgtttttccttcattttaatt

+496 tgggtcgatattttgtttgtattgcaatattcttccggtctgacagattagta TGC
 Cys

+558 GGT GAC ATT CAC GGC CAA TAT TAT GAT CTT ATG GAA ATA TTT GAA
 Gly Asp Ile His Gly Gln Tyr Tyr Asp Leu Met Glu Ile Phe Glu

+597 GTT GGA GGT AAT CCA GCA AAT ACC CAA TAT CTT TTC CTT GGA GAT
 Val Gly Gly Asn Pro Ala Asn Thr Gln Tyr Leu Phe Leu Gly Asp

+641 TAC GTG GAT CGA GGA TAT TTC TCA ATT GAG TGT TTA TTG TAC TTG
 Tyr Val Asp Arg Gly Tyr Phe Ser Ile Glu Cys Leu Leu Tyr Leu

+687 TGG GCT TTG AAA ATA TGG TAT CCG AAA ACG CTT TGG TTG CTT CGT
 Trp Ala Leu Lys Ile Trp Tyr Pro Lys Thr Leu Trp Leu Leu Arg

+732 GGC AAT CAC GAA TGC GCT CAT CTT ACT GAC TAT TTT ACC TTC AAG
 Gly Asn His Glu Cys Ala His Leu Thr Asp Tyr Phe Thr Phe Lys

+777 TTA GAA TGT ACA CAT AAG TAT AAT ATT AAA GTT TAT GAA GCT TGT
 Leu Glu Cys Thr His Lys Tyr Asn Ile Lys Val Tyr Glu Ala Cys

+822 TTA CAA TCT TTT AAT GCC CTT CCT TTG GCA GCT ATT ATG AAT AAG
 Leu Gln Ser Phe Asn Ala Leu Pro leu Ala Ala Ile Met Asn Lys

+867 CAA TTT TTA TGT GTA CAT GGT GGA TTA TCA CCC GAA CTA CAT ACT
 Gln Phe Leu Cys Val His Gly Gly Leu Ser Pro Glu Leu His Thr

+912 TTG AAT GAT ATA CGA ATG ATC AAT CGT TTT TGT GAG CCT CCA ACT
 Leu Asn Asp Ile Arg Met Ile Asn Arg Phe Cys Glu Pro Pro Thr

+957 CAT GGG TTG ATG TGT GAT TTA CTC TGG TCT GAT CCC CTT GAA GAT
 His Gly Leu Met Cys Asp Leu Leu Trp Ser Asp Pro Leu Glu Asp

+1001 TTT GGA TCG GAA AAA TCA AAC AAG CAT TTT ATT CAT AAT AAT GTT
 Phe Gly Ser Glu Lys Ser Asn Lys His Phe Ile His Asn Asn Val

+1047 CGT GGC TGT TCT TAC TTT TAT AGT TAC CAA GCT GTT TGC ACC TTC
 Arg Gly Cys Ser Tyr Phe Tyr Ser Tyr Gln Ala Val Cys Thr Phe

+1092 TTA GAA AAC AAT AAT CTC CTC TCC GTT ATT CGT GCT CAT GAA GCT
 Leu Glu Asn Asn Asn Leu leu Ser Val Ile Arg Ala His Glu Ala

+1137 CAA GAT GTG GGC TAT CGA ATG TAT CGC AAA ACT AAA ACA ACC GGG
 Gln Asp Val Gly Tyr Arg Met Tyr Arg Lys Thr Lys Thr Thr Gly

+1182 TTT CCA TCT TTG ATG ACC ATA TTT TCA GCA CCC AAC TAT TTG GAT
 Phe Pro Ser Leu Met Thr Ile Phe Ser Ala Pro AsnTyur Leu Asp

+1227 GTT TAC AAT AAT AAA GCT GCT GTT TTG AAA TAC GAA AAT AAT GTA
 Val Tyr Asn Asn Lys Ala Ala Val Leu Lys Tyr Glu Asn Asn Val

+1272 ATG AAT ATA AGA CAA TTT AAT TGT TCC CCT CAT CCT TAC TGG CTT
 Met Asn Ile Arg Gln Phe Asn Cys Ser Pro His Pro Tyr Trp Leu
 +1317 CCT AAC TTT ATG GAT GTT TTT ACT TGG AGT CTT CCA TTT GTG GGC
 Pro Asn Phe Met Asp Val Phe Thr Trp Ser Leu Pro Phe Val Gly
 +1362 GAA AAA GTT TCT GAA ATG TTG ATT TCA ATG CTC AAT ATA TGT TCT
 Glu Lys Val Ser Glu Met Leu Ile Ser Met Leu Asn Ile Cys Ser
 +1407 AAG GAG GAG CTT TAT GAA ACG GAT TTG AAA GAA TCG GCT CCG ACT
 Lys Glu Glu Leu Tyr Glu Thr Asp Leu Lys Glu Ser Ala Pro Thr
 +1452 CAG CAT AAA CAG CCT GCT CCA TCT GAG AAT GAA AAT AAA GCC GAC
 Gln His Lys Gln Pro Ala Pro Ser Glu Asn Glu Asn Lys Ala Asp
 +1497 CAA GAA ATT GAT ATT GAA GCT CGT AGG CAA ATT ATT AAA AAC AAG
 Gln Glu Ile Asp Ile Glu Ala Arg Arg Gln Ile Ile Lys Asn Lys
 +1542 ATT ATG GCT ATT GGC CGT ATC TCT CGA GTA TTC AGT GTG CTT AGA
 Ile Met Ala Ile Gly Arg Ile Gly Arg Val Phe Ser Val Leu Arg
 +1587 GAA GAA CGG GAA AGT GTT TCG GAA CTG AAA AAC GTG TCA GGT ACG
 Glu Glu Arg Glu Ser Val Ser Glu Leu Lys Asn Val Ser Gly Thr
 +1632 CAA AGA TTA CCT GCG GGT ACT TTA ATG TTG GGA GCT GAG GGT ATC
 Gln Arg Leu Pro Ala Gly Thr Leu Met Leu Gly Ala Glu Gly Ile
 +1677 AAA AAT GCT ATC AAT TCC TTC GAT GAT GCT CGT AAG CTG GAT ATC
 Lys Asn Ala Ile Asn Ser Phe Asp Asp Ala Arg Lys Leu Asp Ile
 +1722 CAA AAT GAA CGT TTA CCG CCA TCT AAT AGC CGT CGA CGT TCT ACG
 Gln Asn Glu Arg Leu Pro Pro Ser Asn Ser Arg Arg Arg Ser Thr
 +1767 GAT TTA AAG GCT TTC GAA GAA GTA ATG AAT TCG TCT GAA GAT GAT
 Asp leu Lys Ala Phe Glu Glu Val Met Asn Ser Ser Glu Asp Asp
 +1812 ACC TCT ATC GAT CAT TTG GTA GAA CGC TTT GCA GAT AAG AAA AGC
 Thr Ser Ile Asp his Leu Val Glu Arg Phe Ala Asp Lys Lys Ser
 +1857 TCT TTG TAG ataccaaaacctgcgcatattcggttttttagttggaatttttcaat
 Ser Leu
 +1913 gagcataaacctgatccgaggttttaagcatacttacaacaaatatctaaacactcaaa
 +1972 ataaagaaagcaaatgagtttacttttagcataatacaagataaccgatgact 3'

Figure 9. Alignment of the Putative *S. pombe* CnA gene Product with other CnA Homologues.

The *S. pombe* CnA gene product is compared with other CnA homologues. Sequences were aligned using the Sequence Analysis Software Package by Genetics Computer Group (GCG). Identity is shown by “-”.

S.pombe	1	MTSGPHNLEDPVRAIRQKQ...	APSHDITFVQEDGCRVSTLDRVVKVQAPATYIPTDVEFFDINEBDKPDLHFLR
Rat (CNA2)	1	maa...	peparaappppppppppiga-----a-pf-a-hrl-se-v-md..gi-rvdv-k
Human (CNA2)	1	maa...	peparaappppppppppiga-----a-pf-p-hrl-se-v-id..gi-rvdv-k
S.C. (CNA1)	1	mskd-nssrik-i-kpndsyikvdrk-k-l-kyel-n-kv-i-k-pias-p-itgk-s-e-v-.sktql-nhs---	
S.pombe	77	NHFIREGRLSEQTLTIIRKATEILKSEDNLIEVDAPVTVCGDIGHQYYDIMEIFEVGNEPANTQYLFGLGVDRGYFSI	
Rat (CNA2)	57	--lvk---vd--ia-r-negaa--rr-ktm--e-i-----ff--kl-----s-----r-----	
Human (CNA2)	57	--lvk---vd--ia-r-negaa--rr-ktm--e-i-----ff--kl-----s-----r-----	
S.C. (CNA1)	77	e--fh---k--aik-lmms-va-sk-p--lkik-i-i-----kl-----d--eid-----a--f	
S.pombe	157	ECLLYLWALKIWPYKTLMLRGNHECAHLTDYFTFKLECTHKYNIKVEACLOSFNALPLAIMNKQFLCVHGLSPELH	
Rat (CNA2)	137	--v---v---l--s--f-----r-----q--k-i--ser-----mea-ds-----ll-q-----i-	
Human (CNA2)	137	--v---v---l--s--f-----r-----q--k-i--ser-----mea-ds-----ll-q-----i-	
S.C. (CNA1)	157	--i--ys--lmlgrf-m-----k--s-----n-mi--dme--d--cr--v-----l--g-yf-----i--k	
S.pombe	237	TLNDRMINRCEPPTHGLMCDLWSDPLEDEGS.....EKS NKHF IHNVRGCSYFYSAVCTFLENNULSVIRA	
Rat (CNA2)	217	--d---rld--k--af-p-----s-----n.....qe-s--t-----n-p--e--q-----i--	
Human (CNA2)	217	--d---rld--k--af-p-----s-----n.....qe-s--t-----n-p--e--q-----i--	
S.C. (CNA1)	237	sve-vnk--r-l-sr-----a--v-nyddardsefdq--ede-vp-sl---fatfk-s-k--ka-g---i---	
S.pombe	310	HEADVGYRMYRKTCTTGFPSLMTIFSAPNYLDVYNNKAVALKYENNVNMIROFNCSPHPYWLPMFMDVFTWSLPEVGEK	
Rat (CNA2)	290	-----a-----sq-----i-----	
Human (CNA2)	290	-----a-----sq-----i-----	
S.C. (CNA1)	317	-----a-----kn-v-----i-m-----t-h-----e-----hm-----d-----	
S.pombe	390	VSEMLISMLNICKSEELY..ETDLK.ESAPT.....QHKQAPSENNENKADQEIIDEARQIINKKIMAGRISRVF	
Rat (CNA2)	370	-t---vuv-s---dd--mteg-dqfd.vgsaa.....ar-eilrnkairaigkmarvsvl-e.esesvltlkgtpt.	
Human (CNA2)	370	-t---vuv-s---dd--mteg-dqfd.vgsaa.....ar-eilrnkairaigkmarvsvl-e.esesvltlkgtpt.	
S.C. (CNA1)	397	-ts--v-i-----eq--dp..-sep--.aaee-vkaranat-etgtpsd-kassalld-t--kalt--l--akv--m-	
S.pombe	459	SVLREERESVSELKNVSGTQRLPAGTLMIGAEGINNAINSFDDARKLDIQNERLPPSNSRRSTDLKAFEEVVMNSSEDDT	
Rat (CNA2)	440	gm-psgvlaggrtqlq-a-veaieaekairgsspphr-c--ee-kg--ri--m--rkavqdgfnsIntahatenhg-	
Human (CNA2)	439	gm-psgvlaggrtqlq-a-veaieaekairgspbr-c--ee-kg--ri--m--rkavqdgfnsIntahatenhg-	
S.C. (CNA1)	473	-----s-k-ey--lmag.v--r-a-ar-t--lnetlst-ek---e-li--k-----l--evegek-i-yy-kilkga-kkp	
S.pombe	539	SIDHLERFADKKSSL*	
Rat (CNA2)	520	gnhsaq	
Human (CNA2)	519	gnhtaq	
S.C. (CNA1)	552	ql	

Figure 10. Cell Cycle Dependent Expression CnA mRNA.

Levels of CnA mRNA were analyzed during synchronous cell cycle progression as described in Materials and Methods.

(A). The % of binucleated cells was used to monitor synchrony and cell cycle stage. S-phase coincides with the peak of bi-nucleated cells.

(B). CnA mRNA levels at the same time points. The arrows in (A) indicate the peak of mRNA levels.

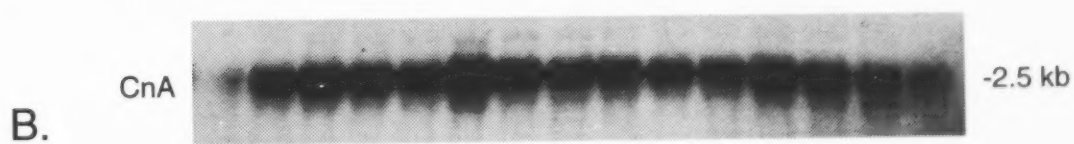
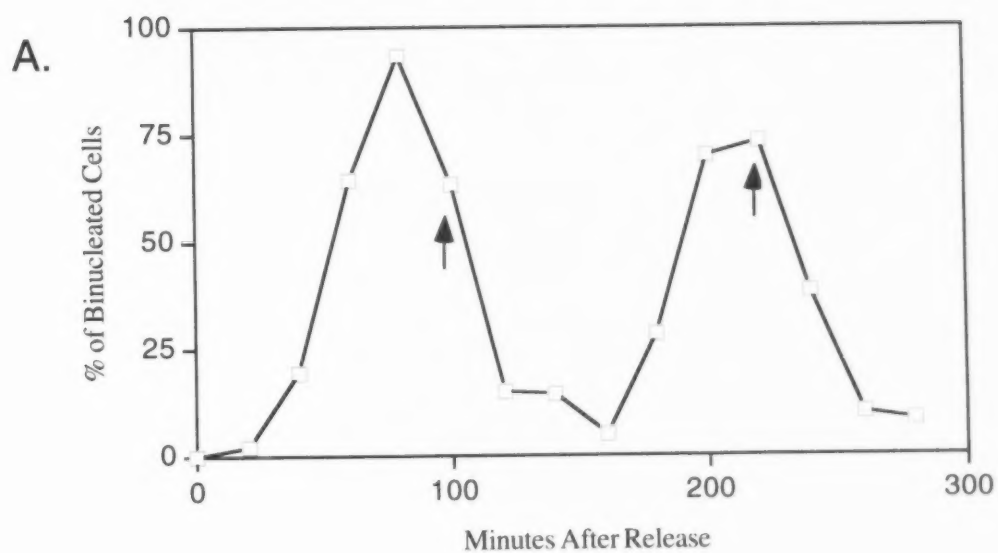


Figure 11. Quantitative Analysis of Nutritional Dependent Expression of CnA mRNA.

The levels of CnA mRNA in cells cultured under different nutritional conditions: mid-exponential (EXP), saturated (SAT), nitrogen deprived (-NIT) and glucose deprived (-GLU) were determined by slot blot hybridization. Serial dilution of equal amounts (starting with 10 ug) of total RNA from described conditions was performed. RNA was immobilized on a Magna Nylon Membrane filter using a Mini-fold slot blot apparatus (Tyler Research) as described in Materials and Methods. The blot was probed with the 2.1 kb EcoRI restriction fragment (see Figure 7) of the *S. pombe* CnA.

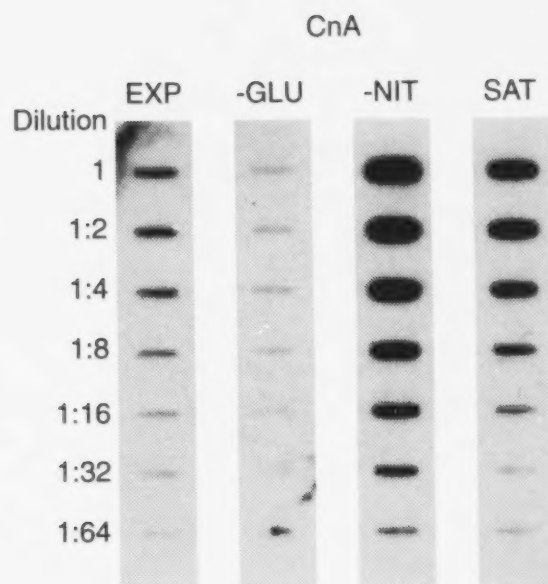


Figure 12. Quantitative Analysis of Nutritional Dependent Expression of Histone H2A mRNA.

Fig. 13 shows the autoradiogram of the same blot used for Fig.12, but stripped and re-probed with the 1.1 kb HindIII restriction fragment of the *S. pombe* histone H2A gene.

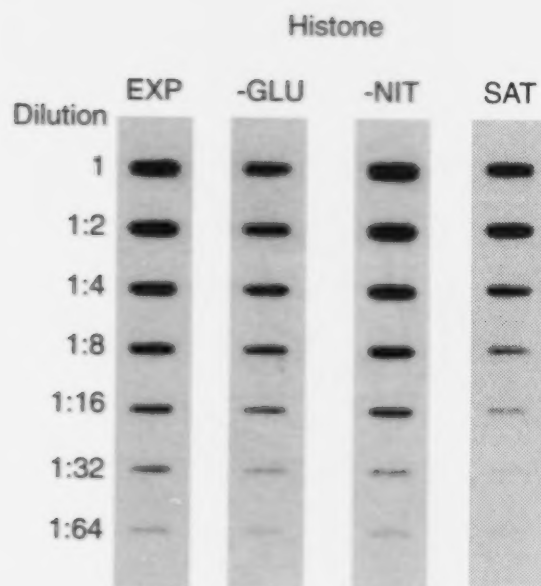


Figure 13. *ste11* Dependent Expression of the *S. pombe* CnA gene.

Time course of nitrogen deprivation dependent induction of CnA mRNA in *S. pombe* was examined by slot blot hybridization. Equal amounts (10ug) of total RNA from the indicated time points were immobilized on a Magna Nylon Membrane filter using a Mini-fold apparatus (Tyler Research), as described in Materials and Methods.

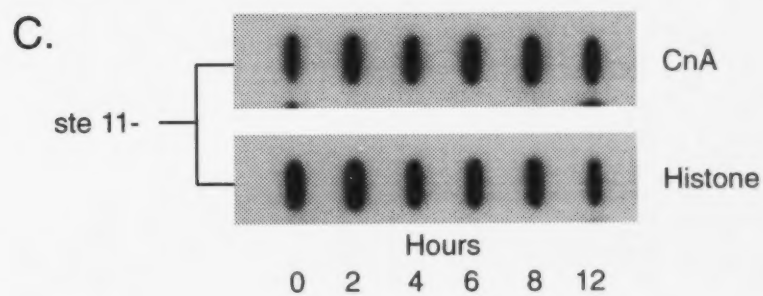
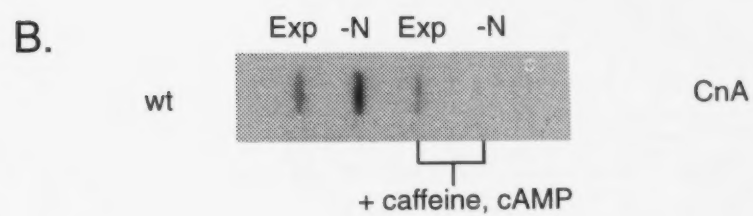
(A). An autoradiogram of the filter probed with the 1.5 kb EcoRI-HindIII restriction fragment of the *S. pombe* CnA gene.

(B). The levels of *S. pombe* CnA mRNA expression after 18 hrs of nitrogen starvation in presence or absence of caffeine and cAMP were examined by slot blot hybridization. The samples were exponentially growing cells (EXP), or nitrogen-deprived (-N).

(C). The levels of CnA mRNA in an *S. pombe* strain lacking the *ste11* gene were examined during nitrogen starvation by slot blot hybridization. The upper panel shows an autoradiogram of the filter probed with 1.5 kb EcoRI-HindIII restriction fragment of the *S. pombe* CnA gene. The lower panel shows an autoradiogram of the same filter stripped and re-probed with 1.1 kb HindIII restriction fragment of the *S. pombe* histone H2A gene.

wt - wild-type cells

ste11- - strain lacking a *ste11* gene



References

- Alfa, C., Fantes, P., Hyams, J., McLeod, M. & Warbrick, E. (1992). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 169.
- Ausubel, F.M., Brent, R., Kingston, R., Moore, D., Seidman, J.G., Smith, J.A. & Struhl, K. (1991). Geerne Publishing, Associates and Wiley-Interscience, N Y.
- Baitinger, C., Alderton, J., Poenie, M., Schulman, H. & Steinhardt, R.A. (1990). *J. Cell. Biol.* 111, 1763-1773.
- Brooks, R.F. (1976). *Nature*. 260, 248-250.
- Chafouleas, J.G., Bolton, W.E., Hidaka, H., Boyd A.E., I. & Means, A.R. (1982). *Cell*. 28, 41-50.
- Chafouleas, J.G., Bolton, W.E., Boyd III, A.E. & Means, A.R. (1984). *Cell*. 36, 73-81.
- Clardy, J. (1995). *Proc. Natl. Acad. Sci. USA*. 92, 56-61.
- Cunningham, K.W. & Fink, G.R. (1994). *J Cell Biol.* 124, 351-363.
- Cyert, M.S., Kunisawa, R., Kaim, D. & Thorner, J. (1991). *Proc. Natl. Acad. Sci. USA*. 88, 7376-7380.
- Cyert, M.S. & Thorner, J. (1992). *Mol. Cell. Biol.* 12, 3460-3469.
- Egel, R. & Egel-Mitani, M. (1974). *Exp. Cell Res.* 88, 127-134.
- Fukui, Y., Kozasa, Y., Kaziro, T., Takeda, Y. & Yamamoto, M. (1986). *Cell*. 44, 329-336.
- Gonzales, G.A. & Montmiry, M.R. (1989). *Cell*. 59, 675-680.

- Guerini, D. & Klee, C.B. (1991). *Proc. Natl. Acad. Sci. USA.* 86, 9183-9187.
- Hanahan, D. (1983). *J. Mol. Biol.* 166, 557-580.
- Hazelton, B., Mitchell, B. & Tupper, J. (1979). *J. Cell Biol.* 83, 487-498.
- Henikoff, S. (1984). *Gene (Amst.)*. 28, 351-359.
- Heichman, K. & Roberts, J. (1994). *Cell.* 79, 557-562.
- Higuchi, S., Tamura, J., Giri, P.R., Polli, J.W. & Kincaid, R.L. (1991). *J. Biol. Chem.* 266, 18104-18112.
- Hoheisel, J., Maier, E., Mott, R., McCarthy, L., Grigoriev, A. & Lehrach, H. (1993). *Cell.* 73, 109-120.
- Issihiki, T., Mochizuki, N. & Maeda, T. (1992). *Genes and Dev.* 6, 2455-2462.
- Ito, A., Hashimoto, T., Hirai, M., Takeda, T., Shuntoh, H., Kuno, T. & Tanaka, C. (1989). *Bioch. Biophys. Res. Commun.* 163, 1492-1497.
- Johnson, K.W., Davis, B.H. & Smith, K.A. (1988). *Proc. Natl. Acad. Sci. USA.* 85, 6072-6076.
- Johnston G.C., P.J.R., Hartwell L.H., (1977). *Exp. Cell. Res.* 105, 79-98.
- Keith, C.H., Ratan, R., Maxfield, F.R., Bajer, A. & Shelanski, M.L. (1985). *Nature.* 316, 848-850.
- Kincaid, R.L., Ploavarapu, R.G., Higuchi, S., Tamura, J., Dixon, S.C., Marietta, C.A., Amorese, D.A. & Martin, B.M. (1990). *J. Biol. Chem.* 265, 11312-11319.
- Kretsinger, R.H. (1980). *CRC Crit. Rev. Biochem.* 8, 119-174.

- Kuno, T., Takeda, T., Hirai, M., Ito, A., Mukai, H. & Tanaka, C. (1989). *Bioch. Biophys. Res. Commun.* 165, 1352-1358.
- Larsson, O., Dafgard, E., Engstrom, W. & Zetterberg, A. (1986). *J. Cell. Physiol.* 127, 267-273.
- Leof, E.B., Wharton, W., O'Keefe, E. & Plenger, W.J. (1982). *J. Cell. Biochem.* 19, 93-103.
- Liu, Y., Ishii, S., Yokai, M., Tsutsumi, S., Ohke, O., Akada, R., Tanaka, K., Tsuchiya, E., Fukui, S. & Miyakawa, T. (1991). *Mol. Gen. Genet.* 227, 52-59.
- Lu, K.P. & Means, A.R. (1993). *End. Rev.* 14, 40-58.
- Matsumoto & Yanagida. (1985). *EMBO.* 4, 3531.
- McCaffrey, P.G., Perrino, B.A., Soderling, T.R. & Rao, A. (1993). *J. Biol. Chem.* 268, 3747-3752.
- Means, A.R., VanBerkum, M.F.A., Bagchi, I., Lu, K.P. & Rasmussen, C.D. (1991). *Pharmac. Ther.* 50, 255-270.
- Means, A. (1994). *FEBS letters.* 347, 1-4.
- Mellow, P.L., Clegg, C.H., Correl, L.A. & McKnight, G.S. (1989). *Proc. Natl. Acad. Sci. USA.* 86, 4887-4892.
- Miyamoto, M., Tanaka, K. & Okayama, H. (1994). *EMBO J.* 13, 1873-1880.
- Mizukami, T., Chang, W.I., Gargaritsev, I., Kaplan, N., Lombardi, D., Matsumoto, T., Niwa, O., Kounousu, A., Yanagida, M., Marr, T.G. & Beach, D. (1993). *Cell.* 73, 121-132.

- Mochizuki, N. & Yamamoto, M. (1992). *Molecular & General Genetics*. 233, 17-24.
- Moreno, S., Klar, A. & Nurse, P. (1991). *Meth. Enzymol.* 194, 795-823.
- Murray, A.W. (1992). *Nature*. 359, 599-604.
- Nakamura, T., Tsutsumi, H., Mukai, H., Kuno, T. & Miyakawa, T. (1992). *FEBS Lett.* 309, 103-106.
- Nakamura, T., Liu, Y.S., Hirata, D., Namba, H., Harada, S., Hirokawa, T. & Miyakawa, T. (1993). *EMBO J.* 12, 4063-4071.
- Neiman, A., Stevenson, B., Xu, H., Sprague, G., Hereskovitz, J., Wigler, M. & Marcus, S. (1993). *Mol. Biol. Cell.* 4, 107-1120.
- Nurse, P. & Bisset. (1981). *Nature*. 292, 558-560.
- Obara-Ishihara, T. & Okayama, H. (1994). *EMBO J.* 13, 1863-1872.
- Ohta, Y., Ohba, T. & Miyamoto, E. (1990). *Proc. Natl. Acad. Sci. USA.* 87, 5341-5345.
- Ohya, Y. & Anraku, Y. (1989). *Curr. Genet.* 15, 113-120.
- Ohya, Y. & Botstein, D. (1994). *Science*. 263, 963-966.
- Okazaki, N., Okazaki, K., Tanaka, K. & Okayama, H. (1991). *Nuc. Acids. Res.* 19, 7043-7047.
- Pardee, A. (1974). *Proc. Natl. Acad. Sci. USA.* 71, 1286-1290.
- Planas-Silva, M.D. & Means, A.R. (1992). *EMBO J.* 11, 507-517.
- Poenie, M., Alderton, J., Tsien, R.Y. & Steinhardt, R.A. (1985). *Nature*. 315, 147-149.

- Rasmussen, C.D. & Means, A.R. (1989). *EMBO J.* 8, 73-82.
- Rasmussen, C., Means, R., Lu, K., May, G. & Means, A. (1990). *J. Biol. Chem.* 265, 13767-13775.
- Rasmussen, C., Garen, C., Brining, S., Kincaid, R.L., Means, R.L. & Means, A.R. (1994). *EMBO J.* 13, 3917-3924.
- Rasmussen, C. & Rasmussen, G. (1994). *Molecular Biology of the Cell.* 5, 784-795.
- Roesler, W.J., Vanderbark, G.R. & Hanson, R.W. (1988). *J. Biol. Chem.* 263, 9063-9066.
- Sambrook, J., Fritsh, E.F. & Maniatis, T. (1989). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sasaki, Y. & Hidaka. (1982). *Biochem. Biophys. Res. Commun.* 104, 451-456.
- Schreiber, S.L. & Crabtree, G.R. (1992). *Immunol. Today.* 13, 135-142.
- Sharma, R.K. & Wang, J.H. (1986). *Biochem. Cell. Biol.* 64, 1072-1080.
- Shoemaker, M.O., Lau, W., Shattuck, R.L., Kwiatkowski, A.P., Matrisian, P.E., Guerra-Santos, L., Lukas, T.J., Van Eldik, L.J. & Watterson, D.M. (1990). *J. Cell Biol.* 111, 1107-1125.
- Stewart, A.A., Ingebritsen, T.S., Manalan, A., Klee, C.B. & Cohen, P. (1982). *FEBS Lett.* 137, 80-84.
- Sugimoto, A., Iino, Y., Maeda, T., Watanabe, Y. & Yamamoto, M. (1991). *Genes & Development.* 5, 1990-1999.
- Sugiyama, A., Tanaka, K., Okazaki, K., Nojima, H. & Okayama, H. (1994). *EMBO J.* 13, 1881-1887.

- Takeda, Y. & Yamamoto, M. (1987). *Proc. Natl. Acad. Sci. USA.* 84, 3580-3584.
- Thorner, J. (1981). Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, NY. 143-180.
- Waldmann, R., Hanson, P.I. & Schulman, H. (1990). *Biochemistry.* 29, 1679-1684.
- Watanabe, Y., Iino, Y., Furuhashi, K. & Shimoda, C. (1988). *EMBO J.* 7, 761-767.
- Whitfield, J.F., MacManus, J.P., Rixon, R.H., Boynton, A.L., Youdale, T. & Swierenga, S. (1976). *In vitro.* 12, 1-18.
- Whitfield, J.F., Boynton, A.L., MacManus, J.P., Rixon, R.H., Sikorska, M., Tsang, B. & Walker, P.R. (1980). *Ann. NY Acad. Sci.* 339, 216-240.
- Ye, R. & Bretsher, A. (1992). *J. Biochem.* 204, 713-723.
- Yoshida, T., Toda, T. & Yanagida, M. (1994). *J. Cell Sci.* 107, 1725-1735.